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Primary Structure and Binding Properties of Calgranulin C, a Novel S100-like Calcium-binding Protein from Pig Granulocytes*

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In this paper we report the biochemical characterization of calgranulin C, a new member of the S100 protein family. The protein is highly abundant in the cytosol of pig granulocytes, with relatively small amounts in lymphocytes. A simple protocol for the rapid purification of calgranulin C is described. The purified protein migrates as a single entity on SDS-polyacrylamide gel electrophoresis while it has two isoforms focusing at pH 5.8 and 5.5. Gel filtration and cross-linking experiments indicate that calgranulin C is capable of dimerization. The complete amino acid sequence was determined by Edman degradation of peptides generated by trypsin and V8 protease digestion. Calgranulin C consists of 91 residues and has a calculated molecular mass of 10,614 daltons. This value is virtually identical to that obtained by electrospray mass spectrometry. Sequence analysis predicts two EF-hand calcium-binding motifs, the first having an extended loop that is distinctive of the S100 protein family. The metal-binding properties were studied by means of a direct $^{45}Ca^{2+}$-binding assay and by tyrosine fluorescence titration. Calgranulin C binds not only calcium but also zinc ions. A single high affinity Zn^{2+}-binding site per monomer was evidenced by fluorimetric titration. Zinc binding to calgranulin C induces a remarkable increase in the protein affinity for calcium; in the absence of zinc, the protein binds 1 Ca^{2+}/monomer with a binding constant of about 2×10^4 M^{-1}, whereas the Zn^{2+}-loaded form binds 2 Ca^{2+}/monomer with K_a values of approximately 3×10^7 and 6×10^4 M^{-1}. Circular dichroism analysis showed that the binding of calcium to calgranulin C induces a 15% decrease in the apparent α-helix content. This result and the calcium-dependent binding of the protein to a phenyl-Superose column strongly suggest that calgranulin C undergoes a gross conformational change upon calcium binding, thus supporting the idea that this protein may be involved in Ca^{2+}-dependent signal transduction events.

Intracellular Ca^{2+} is a ubiquitous second messenger involved in the regulation of many cellular functions (1). The signal is partly transduced into metabolic or mechanical responses by calcium-binding proteins (CaBPs)[1] that interact with cellular effectors in a Ca^{2+}-dependent fashion (2). These proteins include Ca^{2+}/phospholipid-binding proteins of the annexin family (3) and EF-hand CaBPs such as calmodulin, troponin C, and a number of S100 proteins (4, 5). The function of calmodulin in Ca^{2+} signal transduction has been studied extensively, and many target enzymes have been identified (reviewed in Ref. 6). Calcium binding to calmodulin induces a conformational change, thus exposing hydrophobic sites that are involved in the interaction with target proteins (7, 8). The fact that other EF-hand CaBPs also expose hydrophobic regions upon calcium binding (5, 9–12) suggests that this model may represent a general mechanism for the function of these proteins as Ca^{2+} signal mediators.

In granulocytes and monocytes, intracellular Ca^{2+} regulates various acute response activities such as the respiratory burst, phagocytosis, degranulation, and chemotaxis (13–16). Regional increases in Ca^{2+} are thought to occur at sites within the cell where these activities take place (16). Recent efforts to identify calcium signal mediators in granulocytes have led to the discovery of new CaBPs, namely a 33-kDa annexin (17) and a 28-kDa EF-hand protein named grancalcin (18). Additionally, these cells express calmodulin (19) and a heterocomplex formed by two S100 proteins, calgranulins A and B (20, 21).[2]

We have previously reported a preliminary characterization of two abundant CaBPs from pig granulocytes (22). N-terminal sequencing suggested that both proteins belong to the S100 protein family. We identified one of these CaBPs as the porcine counterpart of calgranulin A (22) and proved that, as described for the human and bovine systems (23, 24), it is noncovalently associated with pig calgranulin B (25). Here we focus on the characterization of the other CaBP and demonstrate that it is a new member of the S100 protein family. Its primary structure as well as some binding properties are described. This protein will be referred to as calgranulin C, consistent with the names adopted for other S100 proteins isolated from granulocytes (21, 26).

EXPERIMENTAL PROCEDURES

Materials—$^{45}CaCl_2$ (5 Ci/g) was from Du Pont NEN. Electrophoresis reagents were purchased from Bio-Rad. Dimethyl suberimidate was from Pierce. Sequencing-grade reagents and solvents were obtained from Applied Biosystems. Sequelon-AA™ membranes were from Millipore Corp. PhastGel 4–6.5, molecular mass markers, and pI markers were from Pharmacia LKB (Uppsala, Sweden). Percoll, Sephadex G-75, horse heart myoglobin, bovine serum albumin, trypsin, and V8 protease were purchased from Sigma. All other solvents and reagents were of analytical grade.

Preparation of Lymphocyte and Granulocyte Extracts—Pig lymphocytes and granulocytes were isolated from fresh blood by dextran

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The amino acid sequence reported in this paper has been submitted to the Protein Identification Resource and SWISS-PROT protein sequence data banks with the accession number P80310.

[1] The abbreviations used are: CaBP, calcium-binding protein; ESMS, electrospray mass spectrometry; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase-HPLC; PAGE, polyacrylamide gel electrophoresis; V8 protease, Glu-C-specific endoprotease from *Staphylococcus aureus* strain V8; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; Tricine, *N*-tris(hydroxymethyl)methylglycine.

[2] Synonyms of calgranulins A and B are MRP8 and MRP14, L1 light and heavy chain, p8 and p14, and p7A and p24, respectively.

sedimentation followed by Percoll gradient centrifugation (22) and were more than 95% pure. Cells were suspended in homogenization buffer (10 mM phosphate, 150 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.0), and disrupted in a glass-Teflon homogenizer. The homogenate was centrifuged at 30,000 × *g* for 15 min at 4 °C. The resulting supernatant was centrifuged at 105,000 × *g* for 90 min at 4 °C.

Purification of Calgranulin C—Supernatants from cell extracts (3–4 ml) were fractionated at 4 °C on a Sephadex G-75 column (2.5 × 38 cm) equilibrated with the homogenization buffer. Elution was performed at a flow rate of 15 ml/h. Eluted fractions corresponding to a molecular mass of 10–15 kDa (elution volume: 125–140 ml) were pooled, diluted 3-fold with 20 mM Tris-HCl (pH 9.0), and loaded onto a Mono Q HR 5/5 column (Pharmacia LKB) previously equilibrated with the same buffer. The column was developed on an FPLC system (Pharmacia LKB) with a combination of two linear gradients of NaCl concentration (0–0.14 M in 20 min followed by 0.14–0.35 M in 15 min). The flow rate was 1 ml/min. Calgranulin C eluted at approximately 0.15 M NaCl.

Protein Assay—Protein concentration in crude cell extracts was estimated by Lowry's method (27) with bovine serum albumin as a standard. The concentration of pure calgranulin C was determined by UV absorbance in 6 M guanidine-HCl (28). As the protein has 2 tyrosine residues and no tryptophan (see "Results"), $\Delta\varepsilon_{275\,nm} = 2930\ M^{-1}\ cm^{-1}$ was used (29).

Electrophoresis—SDS-PAGE was performed as described by Schägger and von Jagow (30) in a Mini-PROTEAN II apparatus (Bio-Rad). Isoelectric focusing was carried out in a Phast System (Pharmacia LKB). Densitometric analysis of Coomassie-stained gels was performed in a Dual-Wavelength Chromato Scanner (Shimadzu, Kyoto, Japan).

Cross-linking Experiments—Protein samples in 0.2 M Bicine (pH 8.5) were treated with 1 mM dimethyl suberimidate at 20 °C during 1 h. Subsequently, the reaction was quenched by the addition of glycine to a final concentration of 10 mM, and reaction products were analyzed by SDS-PAGE.

Chromatographic Analysis—Gel filtration analysis of pure calgranulin C (10 μg) was performed by FPLC on a Superose 12 HR 10/30 column (Pharmacia LKB) calibrated with standard proteins. The column was eluted with 50 mM Tris-HCl (pH 7.4) at a flow rate of 0.5 ml/min.

Hydrophobic interaction chromatography was carried out on a phenyl-Superose HR 5/5 column (Pharmacia LKB) equilibrated with 50 mM Tris-HCl (pH 7.4), 0.5 mM $CaCl_2$. After the injection of 30 μg of pure protein, the column was eluted with four column volumes of the same buffer and then with four column volumes of 50 mM Tris-HCl, 1 mM EDTA (pH 7.4).

Mass Spectrometry—The molecular mass of calgranulin C was determined on a VG BioTech/Fisons (Altrincham, United Kingdom) triple-quadrupole instrument equipped with an electrospray ionization source (Analytica). The sample was injected into the ion source in 50% (v/v) methanol and 1% (v/v) acetic acid. Fifteen scans ranging from m/z 800 to 1600 were recorded in each determination. The instrument was calibrated with horse heart myoglobin (average mass 16,951.5 Da).

Enzymatic Digestion and Peptide Purification—The purified protein (400 μg) was digested in 0.1 M ammonium bicarbonate (pH 7.8) with 4 μg of trypsin or 6 μg of V8 protease, at 37 °C during 24 h. Peptides were fractionated by HPLC (Pharmacia LKB) on a Vydac C_{18} column (4.6 × 250 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid in water. Elution was performed at a flow rate of 0.8 ml/min with a 0–80% acetonitrile linear gradient in 100 min.

Amino Acid Analysis and Sequencing—Amino acid analysis was performed on a model 420A amino acid analyzer (Applied Biosystems). Amino acid sequencing was carried out on an Applied Biosystems model 477A protein sequencer equipped with an on-line model 120A phenylthohydantoin analyzer. The intact protein, as well as most of the peptides, was loaded onto a Polybrene-coated glass filter and sequenced according to the manufacturer's instructions. Peptides V7–V10 were covalently bound to a Sequelon-AA[TM] membrane. In this case, sequences were run basically as described by Admon and King (31).

Sequence Comparison—Multiple sequence alignment and phylogenetic tree construction were carried out by using the Darwin system (32).

Calcium Binding Assay—Apo-calgranulin C was prepared by incubation of freshly purified protein with 2 mM EGTA and 2 mM EDTA and subsequent dialysis against 25 mM Tris-HCl (pH 7.4), or by extensive dialysis against 10 mM EDTA (pH 7.4) and then against Milli Q water (Millipore Corp.) (33).

$^{45}Ca^{2+}$ binding was determined as per the method of Moni and Kay (11) that uses microconcentrators as ultrafiltration devices to perform rapid flow dialysis. In short, apo-calgranulin C (15–50 μM) was incubated in a prewashed Centricon 3 microconcentrator (Amicon) with known amounts of $^{45}CaCl_2$ at 20 °C during 5 min and then the sample was centrifuged at 3000 rpm for 5 min. Free $^{45}Ca^{2+}$ concentration was determined by measuring the radioactivity in the filtrate. Each determination was performed at least in triplicate.

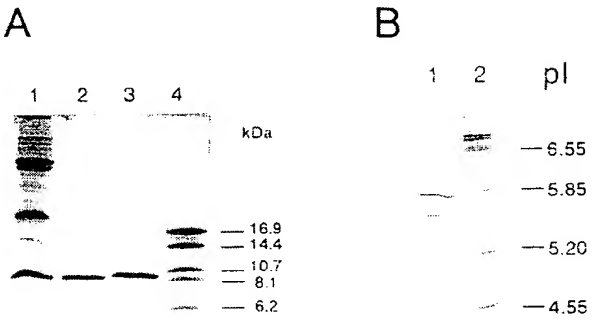

FIG. 1. *A*, SDS-PAGE analysis of calgranulin C-containing samples at different stages of purification. The purification procedure is described under "Experimental Procedures." *Lane 1*, 105,000 × *g* supernatant from pig granulocytes; *lane 2*, after Sephadex G-75 gel filtration; *lane 3*, after Mono Q chromatography; *lane 4*, molecular mass standards. *B*, isoelectric focusing of purified calgranulin C on a PhastGel IEF 4–6.5. *Lane 1*, calgranulin C; *lane 2*, pI standards.

Binding data were analyzed by means of the following equations, where r is the number of moles of calcium bound per mol of monomer, X is free calcium concentration, n is the number of binding sites per monomer, and K_a, K_{a_1}, and K_{a_2} are macroscopic binding constants.

$$r = \frac{n K_a x}{1 + K_a x} \qquad \text{(Eq. 1)}$$

$$r = \frac{\frac{n}{2} K_{a_1} x + n K_{a_1} K_{a_2} x^2}{1 + K_{a_1} x + K_{a_1} K_{a_2} x^2} \qquad \text{(Eq. 2)}$$

Curve-fitting was made by nonlinear regression (SigmaPlot 4.10, Jandel Corp.). The calculated parameters are expressed as mean ± S. E.

Fluorescence Measurements—Tyrosine fluorescence was registered at 25 °C on a Jasco FP-770 spectrofluorometer (Japan Spectroscopic Co., Hachioji City, Japan). The excitation wavelength was set to 278 ± 5 nm. Each spectrum represents an average of five scans. For titration experiments, the emission wavelength was set to 308 ± 3 nm. The fluorescence intensity (F) was corrected for sample dilution, the latter never exceeding 3%. Data from titration with calcium in the absence of zinc were fitted to Equation 3, where F_o is the fluorescence at zero ligand concentration, F_m is the maximum fluorescence change, T is the total ligand concentration, P is the protein monomer concentration, and K_a is the apparent association constant.

$$F = F_o + \frac{F_m}{2P}\left(T + P + \frac{1}{K_a} - \sqrt{\left(T + P + \frac{1}{K_a}\right)^2 - 4PT}\right) \qquad \text{(Eq. 3)}$$

This equation is derived from Eq. 1 by setting n to 1 and substituting $[F - F_o)\cdot F_m^{-1}]$ for r and $[T - P\cdot F_m^{-1}\cdot(F - F_o)]$ for X.

Circular Dichroism—Far-UV circular dichroism spectra were obtained on a Jasco J-20 spectropolarimeter calibrated with (+)-10-camphorsulfonic acid. Each spectrum represents an average of four scans. The α-helix content was calculated as described by Zhong and Johnson (34).

RESULTS

Purification of Calgranulin C—The 105,000 × *g* supernatant from pig granulocytes was fractionated on a Sephadex G-75 column. Calgranulin C was recovered as a major component in the 10–15-kDa fraction (Fig. 1*A*, *lane 2*) and was further purified by anion exchange on a Mono Q column. The protein eluted from the column in a symmetric peak and was considered homogeneous per SDS-PAGE (Fig. 1*A*, *lane 3*) and N-terminal sequence. Calgranulin C could also be purified from lympho-

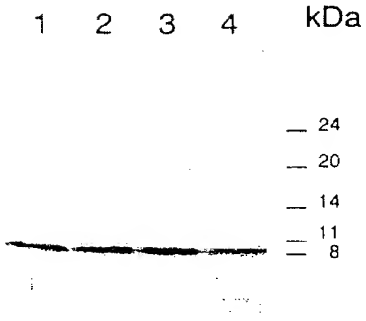

FIG. 2. **Dimer formation of calgranulin C *in vitro*.** The purified protein was analyzed by 16% SDS-PAGE without cross-linking (*lane 1*) and after cross-linking with 1 mM dimethyl suberimidate in the presence of 1 mM EDTA (*lane 2*), 1 mM $CaCl_2$ (*lane 3*), and 0.1 mM $ZnCl_2$ (*lane 4*).

cyte extracts by the same procedure, and its electrophoretic mobility, UV absorption spectrum, and N-terminal sequence (6 cycles) were indistinguishable from those of the protein isolated from granulocytes (data not shown). However, the amounts of calgranulin C obtained from the granulocyte and lymphocyte extracts were 8% and 0.14%, respectively of the total soluble proteins. Thus, the content of calgranulin C was 50–60 times higher in granulocyte than in lymphocyte extracts.

Biochemical Properties of Calgranulin C—The protein migrates on Tricine SDS-PAGE as a 9-kDa polypeptide (Fig. 1*A*, *lane 3*). As some calcium-binding proteins have aberrant mobilities on SDS-PAGE gels (18, 24, 35), a more precise determination of the molecular mass of calgranulin C was made by ESMS. The mass spectrum showed the presence of two components of 10,614 ± 3 and 10,654 ± 3 Da (mean ± S.D.), the second accounting for about 20% of the molecules. This difference in mass (40 Da) could correspond to a calcium atom bound to the molecule, although alternative explanations such as partial N-terminal acetylation (42 Da) should not be ruled out.

Analysis of the purified protein by Superose 12 gel filtration showed two peaks of an apparent molecular mass of 11 and 18 kDa, thus suggesting that the native protein exists both as a monomer and as a homodimer. This conclusion was further supported by cross-linking experiments. Upon treatment with dimethyl suberimidate and subsequent SDS-PAGE analysis, a new protein band of 20 kDa was observed in addition to that of the remaining monomer (Fig. 2, *lane 2*). The presence of either 1 mM $CaCl_2$ or 0.1 mM $ZnCl_2$ during the cross-linking reaction did not modify the dimer/monomer ratio (Fig. 2).

Two calgranulin C forms of pI 5.8 and 5.5 were observed by native isoelectric focusing (Fig. 1*B*). As inferred from densitometric scanning, the relative contents of pI 5.8 and 5.5 isoforms are about 75% and 25%, respectively. This proportion remained unchanged in samples incubated with either 2 mM $CaCl_2$ or 2 mM EDTA before isoelectric focusing. In addition, both forms were observed by denaturing isoelectric focusing (data not shown). Therefore, the charge heterogeneity of calgranulin C is not a consequence of ligand binding.

Primary Structure of Calgranulin C—The purified protein was desalted by RP-HPLC and submitted to N-terminal sequencing. The sequence of the first 40 residues was obtained, except for amino acids at positions 33 and 38, which could not be unambiguously identified. In order to complete the amino acid sequencing of calgranulin C, fragments were generated by enzymatic cleavage of the purified protein. Both tryptic and V8 protease peptides were separated by RP-HPLC and subsequently submitted to amino acid analysis and/or Edman degradation.

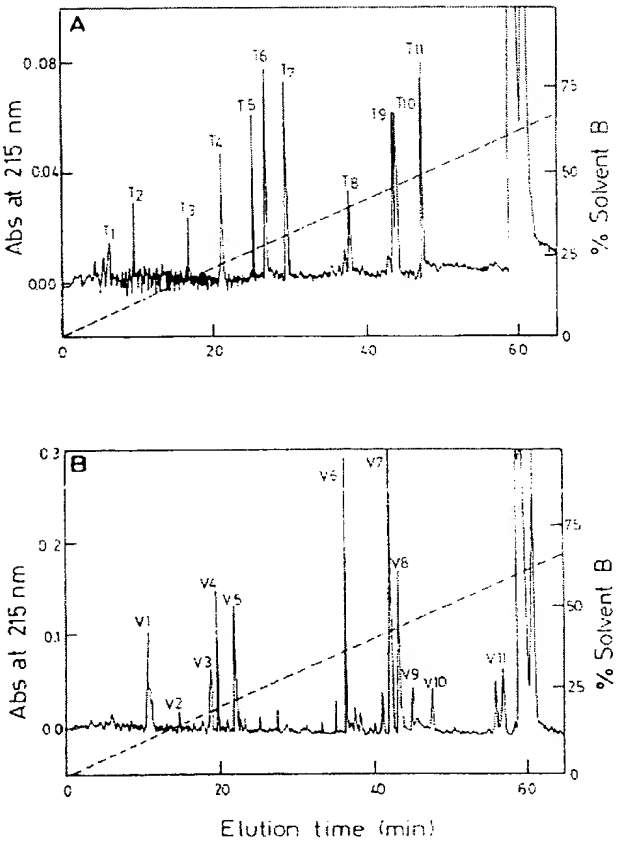

FIG. 3. **HPLC separation of peptides obtained by tryptic (*A*) and V8 protease (*B*) digestion of calgranulin C.** Details of the procedure are described under "Experimental Procedures." *Peaks T1–T11* and *V1–V11* represent peptides whose sequence was determined by Edman degradation or inferred from amino acid analysis. Solvent A: 0.1% trifluoroacetic acid; solvent B: 80% acetonitrile, 0.1% trifluoroacetic acid. No peaks were detected beyond 60% of solvent B.

The tryptic map of calgranulin C is shown in Fig. 3*A*. The peaks identified in the figure (designated T1–T11) represent pure peptides. Except for T6, T8, and T10, their amino acid sequences were determined by Edman degradation. On the basis of their amino acid composition, peptides T6 and T10 were assigned to fragments 21–29 and 1–20, respectively. The sequence of peptide T8 was partially obtained by Edman degradation, the rest being inferred from amino acid analysis. Considering the specificity of trypsin, it should be mentioned that the C-terminal residues of T9 and T11 were Tyr and Glu, respectively, instead of Arg or Lys as expected; the sequence of T9 was identical to that of residues 1–17 and was assumed to originate from a residual chymotryptic activity, while peptide T11 was assigned to the C-terminal end of the protein.

Fig. 3*B* shows the HPLC separation of fragments obtained by digestion with V8 protease. Again, the peaks identified in the figure represent pure peptides. Except for V6, V9, and V11, they were completely sequenced by Edman degradation.

A summary of the sequence analyses and the resulting primary structure of calgranulin C are shown in Fig. 4. The following peptides, all corroborating the proposed sequence, were not included in the figure (residue positions given in parentheses): T6 (21–29), T10 (1–20), V1 (5–8), V2 (1–4), and V9 (5–31). The protein consists of 91 residues and lacks cysteine, methionine, and tryptophan. On the basis of this sequence, the

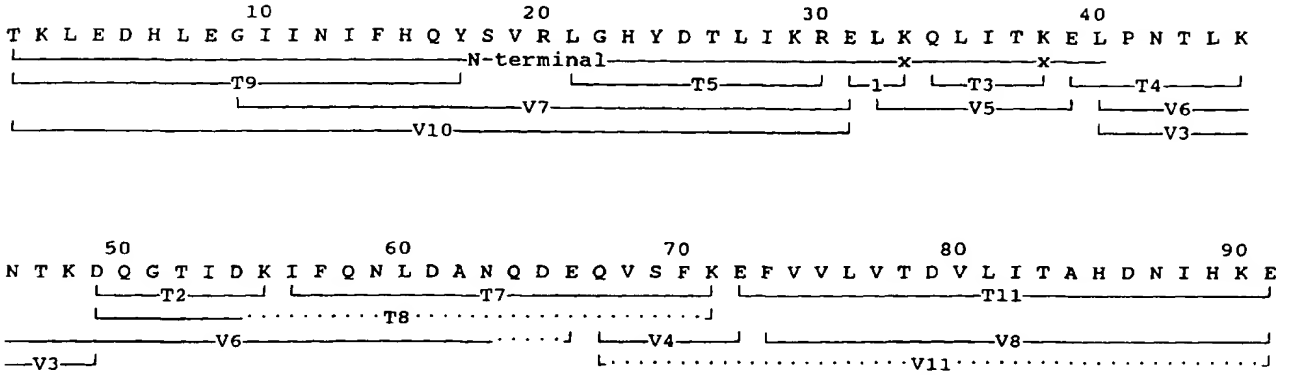

FIG. 4. **Primary structure of calgranulin C.** The nomenclature of peptides is consistent with that of Fig. 3 (*A* and *B*). *X* represents a residue which could not be identified on this particular run. Segments determined by Edman degradation or inferred from amino acid analysis are indicated with *solid* and *dotted lines*, respectively.

molecular mass was calculated to be 10,614 daltons. This value is virtually identical to that obtained by ESMS, thus indicating that the protein has been fully sequenced. The calculated isoelectric point (6.0) agrees with that obtained experimentally for the major calgranulin C isoform (5.8). The amino acid sequence predicts two EF-hand calcium-binding sites (36). The N-terminal site has a 14-residue loop (Ser^{18}–Glu^{31}) that is unique for S100 proteins, while the loop of the C-terminal EF-hand comprises 12 residues (Asp^{61}–Glu^{72}). The hydropathy plot (37) of calgranulin C predicts two hydrophobic regions near the N- and C-terminal ends (data not shown), a feature shared by the S100 proteins (4).

Direct Calcium Binding Studies—The Ca^{2+}-binding isotherm at 20 °C of calgranulin C in 25 mM Tris-HCl (pH 7.4) is shown in Fig. 5 (*closed circles*). Only one Ca^{2+}-binding site per monomer was titrated with free calcium concentrations of up to 0.6 mM. The best fit of the binding data with Equation 1 was obtained with $K_a = 1.9 \pm 0.4 \times 10^4\ M^{-1}$ and $n = 1.10 \pm 0.06$. Due to the experimental limitations of the method at higher ligand concentrations, the existence of an additional low affinity site ($K_a < 10^3\ M^{-1}$) could be neither proven nor ruled out.

As shown in Fig. 5, the presence of zinc ions induces a remarkable increase in the calgranulin C affinity for calcium. The binding isotherm obtained in the presence of 0.1 mM $ZnCl_2$ (*open circles*) was fitted to Equation 2 with the following parameters: $K_{a_1} = 2.7 \pm 0.3 \times 10^7\ M^{-1}$, $K_{a_2} = 6.5 \pm 1.2 \times 10^4\ M^{-1}$, and $n = 2.10 \pm 0.04$.

Tyrosine Fluorescence Titration—The intrinsic emission spectrum of apo-calgranulin C and those of the protein with Ca^{2+}, Mg^{2+}, and Zn^{2+} are shown in Fig. 6*A*. While tyrosine fluorescence was minimally influenced by 5 mM Mg^{2+}, a 7% decrease and a 40% increase were observed upon addition of 2 mM Ca^{2+} and 0.1 mM Zn^{2+}, respectively. Titration with Ca^{2+} in the absence of other metal ions showed the presence of a single class of site with $K_{a(app)} = 2.9 \pm 0.4 \times 10^4\ M^{-1}$ (Fig. 6*B*, *closed circles*), in agreement with direct binding experiments. The presence of 5 mM Mg^{2+} (*open circles*) caused a very slight decrease in the affinity for Ca^{2+} ($K_{a(app)} = 2.3 \pm 0.3 \times 10^4\ M^{-1}$), thus suggesting that the titrated site is highly selective for calcium.

A biphasic curve was obtained when calgranulin C was titrated with Ca^{2+} in the presence of 0.1 mM Zn^{2+} (Fig. 6*C*, *closed diamonds*). The curve was not noticeably influenced by 5 mM Mg^{2+} (*open diamonds*). Analysis of the Ca^{2+}-induced fluorescence change as a function of the fractional Ca^{2+} occupancy (νCa^{2+}), calculated with the previously determined binding constants, indicates that both the increase and decrease in tyrosine fluorescence are concomitant with the binding of calcium to the high and low affinity sites, respectively.

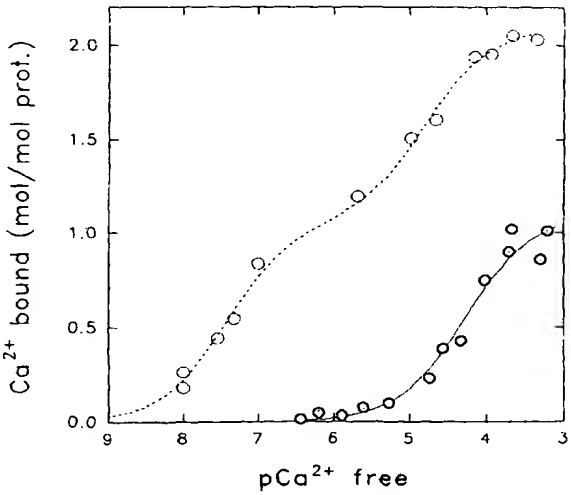

FIG. 5. **Ca^{2+}-binding isotherms of calgranulin C in the absence (*closed circles*) and in the presence (*open circles*) of 0.1 mM Zn^{2+}.** Details of the procedure are described under "Experimental Procedures." The curves depicted by *solid* and *dashed lines* were calculated by fitting Equations 1 and 2 to the experimental points, respectively. Values for the parameters are indicated in the text.

Fluorescence titration was also applied to study the binding of zinc to calgranulin C. As shown in Fig. 6*D*, fluorescence intensity increased almost linearly with added Zn^{2+} up to a ligand/protein monomer molar ratio of 1.0 ± 0.1, thus suggesting the presence of one high affinity Zn^{2+}-binding site per calgranulin C monomer. An almost identical titration curve was obtained in the presence of 0.1 mM Ca^{2+} (data not shown). Although the binding constant for Zn^{2+} could not be accurately calculated from these titration curves, simulations of the experimental data by means of Equation 3 (not shown) indicate that $K_{a(app)}$ should exceed $10^8\ M^{-1}$.

Calcium-induced Conformational Changes—Typical far-UV CD spectra of the apo- and Ca^{2+}-loaded forms of calgranulin C are shown in Fig. 7. The calcium-induced change in the CD spectrum may be attributed to a decrease in the overall α-helix content. Analysis of CD data according to Zhong and Johnson (34) indicated apparent α-helix contents of 52% and 44% for the apo- and Ca^{2+}-loaded forms, respectively.

Calcium binding also affected the chromatographic behavior of calgranulin C on a phenyl-Superose column. The protein was entirely bound to the column in the presence of 0.5 mM $CaCl_2$

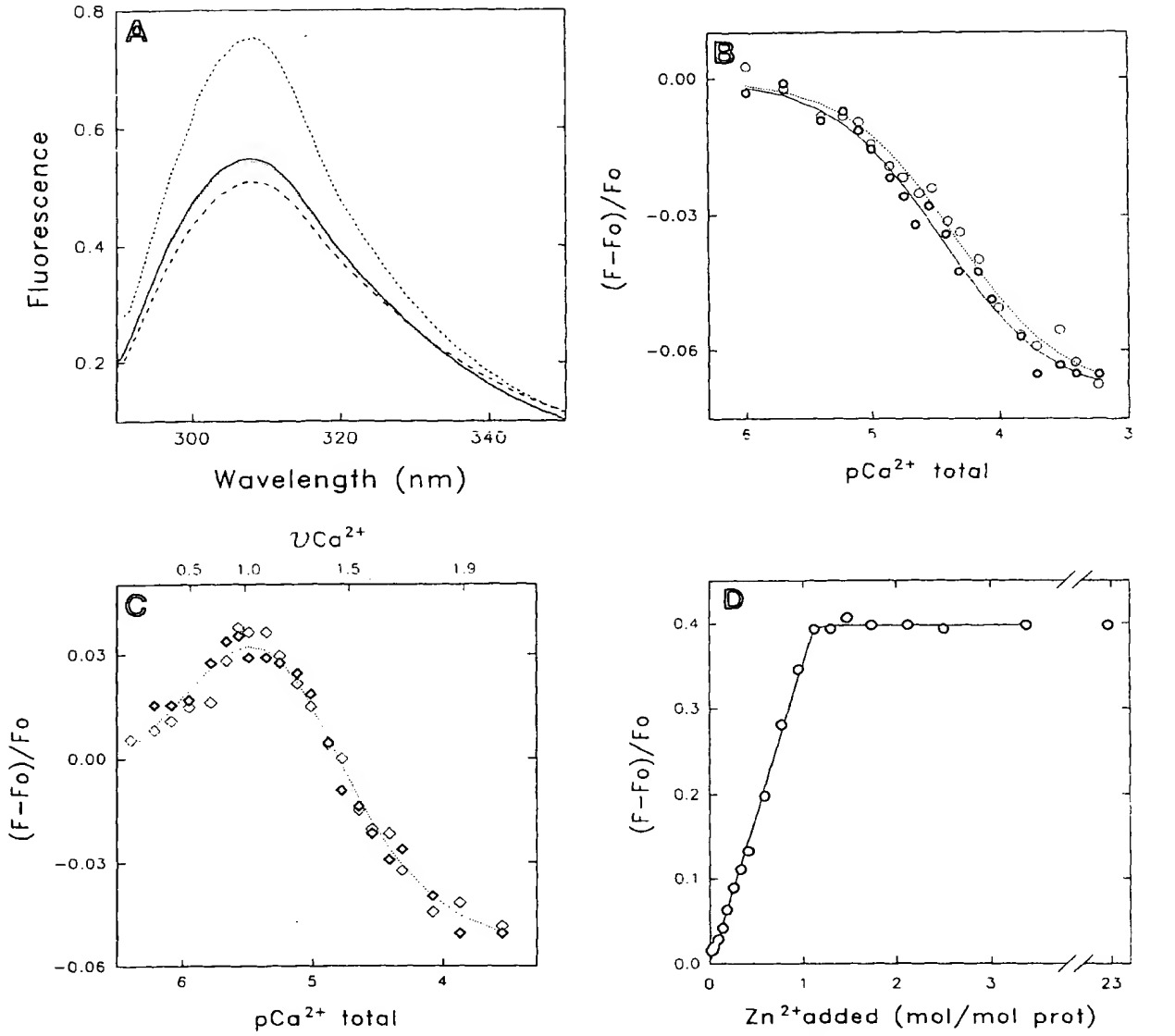

FIG. 6. *A*, fluorescence spectra of calgranulin C (10 μM) in 25 mM Tris-HCl (pH 7.4) with either 2 mM EDTA (*solid line*), 5 mM $MgCl_2$ (*dotted line*), 2 mM $CaCl_2$ (*long-dashed line*), or 0.1 mM $ZnCl_2$ (*short-dashed line*). *B–D*, tyrosine fluorescence titration experiments. Protein concentration was 2.2 μM. In each experiment, values of fluorescence intensity at 308 nm (F) were normalized to those corresponding to zero ligand concentration (F_o). *B*, titration of apo-calgranulin C with Ca^{2+} in the absence (*closed circles*) or in the presence (*open circles*) of 5 mM $MgCl_2$. The curves were calculated by fitting Equation 3 to the experimental points. *C*, titration of Zn^{2+}-bound calgranulin C with Ca^{2+} in the absence (*closed diamonds*) or in the presence (*open diamonds*) of 5 mM $MgCl_2$. The top of the panel indicates the corresponding amount of Ca^{2+} bound per mol of calgranulin C monomer (νCa^{2+}) as calculated from the binding data of Fig. 5. *D*, titration of apo-calgranulin C with Zn^{2+}.

and could be eluted from the column with 1 mM EDTA (data not shown).

DISCUSSION

In this paper we report the characterization of calgranulin C, a new member of the S100 protein family. Members of this family are acidic CaBPs about 100 residues in length. They contain two EF-hand motifs per monomer, the first having an unusual 14-residue calcium-binding loop that is distinctive of this family (2, 38). Most S100 proteins are expressed in a tissue-specific and cell cycle-specific fashion, this leading to the proposal that they are involved in cell differentiation and cell cycle progression (4, 39–41). Moreover, some S100 proteins such as CACY and CAPL are associated with tumor development and the induction of metastasis (42, 43). Other functions postulated for S100 proteins include the regulation of cytosolic Ca^{2+} concentration, inhibition of specific phosphorylation events, and modulation of cytoskeletal-membrane interactions (4, 44, 45).

Calgranulin C was purified from pig granulocytes by a simple procedure involving gel filtration and anion exchange chromatography. As judged from the amount of pure protein obtained by this method, calgranulin C comprises at least 8% of pig granulocyte cytosolic proteins. This percentage could be even higher since losses inherent in the chromatographic steps were not taken into account. The protein was also purified from lymphocyte extracts,

although the amount of pure calgranulin C obtained per mg of total protein was 50–60 times smaller than that obtained from granulocytes. In fact, this low content of calgranulin C in the lymphocyte extracts can be explained by the presence of contaminating granulocytes (usually 1–3%) in the lymphocyte preparations used. Therefore, there is a possibility that pig lymphocytes may not express calgranulin C at all.

Fig. 8*A* shows the multiple sequence alignment of the S100 protein family. The amino acid identity between calgranulin C and the other S100 proteins ranges from 27% (S100E) to 45% (calgranulin B). The least conserved segments are the C-terminal region and the "hinge" region that connects the two EF-hands (Fig. 8*A*). These regions are thought to provide specificity to the function of each S100 protein (4). The phylogenetic tree of the S100 family is shown in Fig. 8*B*. Calgranulin C appears to be most closely related to calgranulin B, a protein that is also expressed in granulocytes. However, calgranulin B forms tightly associated heterocomplexes with calgranulin A (23–25) whereas no such heterocomplex formation is observed for calgranulin C. Furthermore, the unusually long C-terminal "tail" characteristic of calgranulin B is absent in calgranulin C (Fig. 8*A*). It is worth mentioning that this "tail" is phosphorylated upon neutrophil activation (24, 46) and that such a phosphorylation event is thought to be important for the function of calgranulin B (41). These structural differences between calgranulins B and C suggest that these proteins may have separate functions in granulocytes.

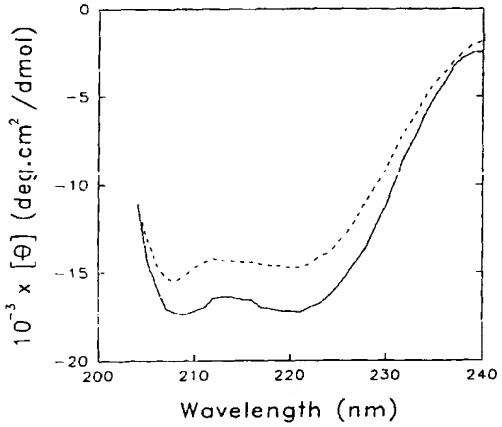

FIG. 7. **Far-UV circular dichroism of calgranulin C.** Spectra were obtained in 50 mM Tris-HCl (pH 7.4) in the presence of either 2 mM EDTA (*solid line*) or 2 mM $CaCl_2$ (*dashed line*). Protein concentration was 50 μM.

Although purified calgranulin C appeared homogeneous by SDS-PAGE and N-terminal sequencing, its ESMS spectrum showed the presence of two components. The molecular mass of the major component (10,614 Da) fits the value calculated from the primary structure, while the minor one is approximately 40 Da heavier. As many EF-hand CaBPs retain their ligand during purification (47), it is likely that this difference in mass is due to a single Ca^{2+} bound to the protein. Isoelectric focusing analysis of calgranulin C also shows the presence of two components, although in this case analyses performed in the presence of Ca^{2+}, EDTA and denaturing agents strongly suggest that the charge heterogeneity is not a consequence of ligand binding. Further experiments will be required to elucidate this point.

Most S100 proteins are known to exist as dimers, and both disulfide-bound (35, 48) and noncovalently associated forms (9, 23) have already been described. Cross-linking experiments and gel filtration analysis of purified calgranulin C demon-

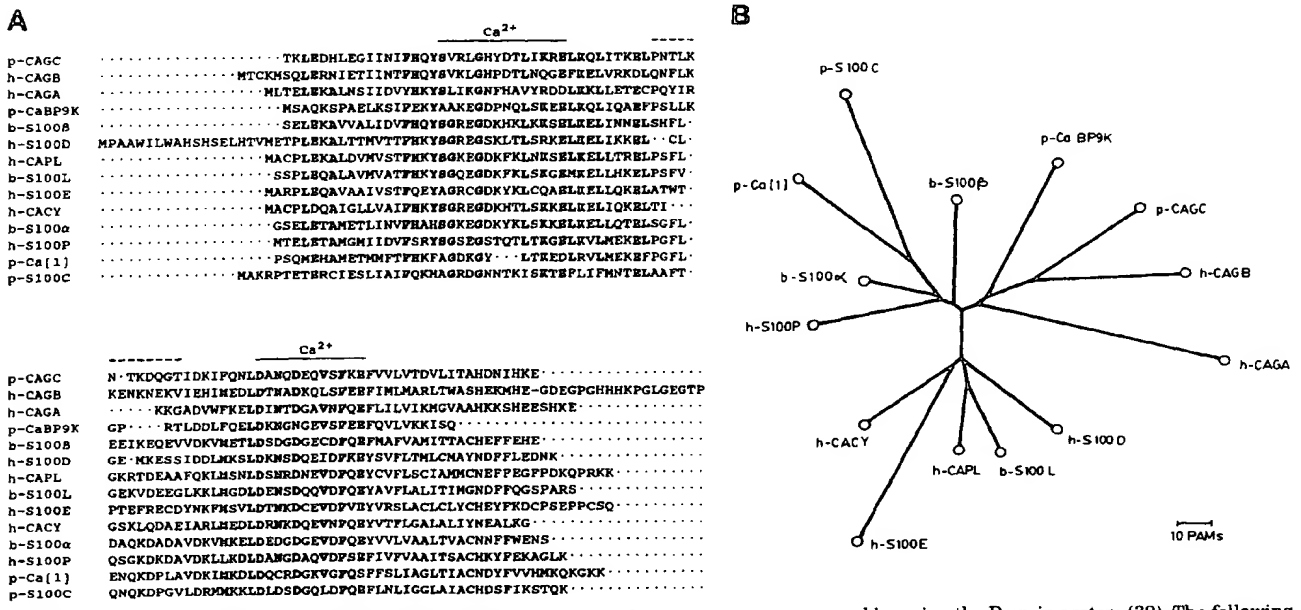

FIG. 8. **Sequence analysis of the S100 protein family.** Amino acid sequences were compared by using the Darwin system (32). The following sequences were obtained from the SWISS-PROT protein data bank (accession codes given in parentheses): *h-CAGB*, human calgranulin B (P06702); *h-CAGA*, human calgranulin A (P05109); *p-CaBP9K*, pig calbindin-D9K (P02632); *b-S100β*, bovine S100 protein β-chain (P02638); *h-CAPL*, human placental calcium-binding protein (P26447); *b-S100L*, bovine S100L (P10462); *h-CACY*, human calcyclin (P06703); *b-S100α*, bovine S100 protein α-chain (P02639); *h-S100P*, human S100P (P25815); *p-Ca[1]*, pig calpactin I light chain (P04163). The other sequences are: *p-CAGC*, pig calgranulin C (this work); *h-S100D*, human S100D (38); *h-S100E*, human S100E (38); *p-S100C*, pig S100C (54). *A*, multiple sequence alignment. The calcium-binding loops of the two EF-hand motifs and the segment connecting both motifs are indicated with *solid* and *dashed lines*, respectively. Residues common to at least 10 sequences are shown in *bold* characters. *B*, phylogenetic tree. The branch lengths are in PAMs (accepted point mutations per 100 residues).

strate the presence of a homodimeric form apart from the monomer. The fact that the amino acid sequence of calgranulin C contains no cysteine residues indicates that the homodimer is noncovalently associated. Whether the dimeric or monomeric forms occur *in vivo* remains to be established.

The binding properties of calgranulin C were studied by means of a direct $^{45}Ca^{2+}$-binding assay and by tyrosine fluorescence titration. Our results indicate that both Ca^{2+} and Zn^{2+} are bound to calgranulin C and that the binding of Zn^{2+} induces a profound change in the Ca^{2+}-binding properties of the protein. In the absence of zinc, the protein appears to bind 1 Ca^{2+}/monomer with a K_a of approximately 2×10^4 M^{-1}. In contrast, Zn^{2+}-loaded calgranulin C binds 2 Ca^{2+}/monomer with stoichiometric binding constants in the order of 10^7 and 10^4 M^{-1}, respectively. The binding of 2 Ca^{2+}/calgranulin C monomer is consistent with the presence of two EF-hand motifs in the amino acid sequence. Both EF-hands seem to be specific for Ca^{2+} as the fluorescence titration curves were minimally affected by 5 mM Mg^{2+}. As also inferred from tyrosine fluorescence titration, calgranulin C has an additional binding site with high affinity for Zn^{2+}. It is worth noting that the C-terminal region of calgranulin C contains a His-*X*-*X*-*X*-His motif comprising residues 85–89 (Fig. 4). This motif within an α-helix has two correctly positioned imidazoles that can chelate a zinc ion (49). As the secondary structure prediction by means of the PHD algorithm (50) suggests that residues 85–89 are within an α-helix (not shown), we propose that the side chains of both His^{85} and His^{89} may participate in the binding of the zinc ion.

The ability of an S100 protein to bind Zn^{2+} with high affinity and the zinc-induced increase in calcium-binding affinity have been previously described only for S100$\beta\beta$ (33). As proposed for this protein, calgranulin C should be considered both as a calcium- and zinc-binding protein. Whether Ca^{2+} binding to calgranulin C is regulated *in vivo* by Zn^{2+} remains to be determined.

The function of calgranulin C in granulocytes is unknown. In addition to its possible role as a Ca^{2+} buffer due to its high concentration, it may be involved in specific calcium-dependent signal transduction pathways. According to the currently accepted mechanism of action of EF-hand CaBPs in Ca^{2+} signal transduction (4, 5), calgranulin C should undergo Ca^{2+}-dependent conformational changes responsible for the transmission of information to effector proteins. Such conformational changes were reported for some members of the S100 protein family, namely S100$\alpha\beta$, S100$\beta\beta$, and S100P (9, 12, 47). In contrast, calbindin-D9K, which has been suggested to act merely as a Ca^{2+} buffer (51), undergoes only very subtle conformational changes upon calcium binding (52). These considerations prompted us to investigate whether the conformation of calgranulin C is affected by calcium. Our results indicate a Ca^{2+}-induced change in the environment of at least 1 of the 2 tyrosine residues of the molecule. Moreover, binding of Ca^{2+} to the protein causes a significant decrease in the apparent α-helix content, which is certainly in line with previous observations on other S100 proteins (9, 11, 47). Finally, the fact that Ca^{2+}-loaded calgranulin C is retained on a phenyl-Superose column and can be eluted with EDTA suggests that the protein exposes a hydrophobic region in the presence of calcium. Taken together, these results demonstrate that calgranulin C undergoes a gross conformational change upon calcium binding and support the possibility that this novel protein is involved in Ca^{2+}-dependent signal transduction events.

Intracellular calcium levels modulate many phagocyte functions including chemotaxis, phagocytosis, degranulation, and the generation of reactive oxygen species (13–16). In addition to calgranulin C, various putative mediators of the calcium signal in granulocytes have been identified, namely calmodulin (19), a 33-kDa annexin protein (17), grancalcin (18), and the calgranulin A/B heterocomplex (53). The notion that each protein may transmit the calcium signal to a different cellular effector deserves future investigation. Ongoing studies are aimed at identifying the cellular target of calgranulin C.

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